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Investigation of the vasorelaxant properties of Nitroxyl during altered glycaemic conditions

Kimberley Sams

Project Advisor: Kathryn Yuill, School of Biomedical and Biological Sciences, Plymouth University, Drake Circus, Plymouth, PL4 8AA

Abstract

Nitroxyl (HNO) is currently a vasorelaxant of high interest, but as yet its mechanisms of action have not been fully elucidated. It is thought that HNO acts on K_V channels to mediate vasorelaxation, and this has been shown in previous studies (Irvine *et al*, 2003; Favarolo & Kemp-Harper, 2009; Yuill *et al*, 2011). It has also been shown that high levels of glucose inhibit K_V channels (Rainbow *et al*, 2006) so would inhibit HNO mediated relaxation. This study could provide further evidence of K_V involvement of HNO mediated vasodilation.

This study uses a temperature controlled organ bath with aortic ring preparations obtained from male Wistar rats, which were killed using approved schedule 1 methods. The section of aorta was connected to an isometric transducer linked to LabChart software. This was used to measure changes in tension in response to the addition of increasing concentrations of sodium nitroprusside (SNP) and Angeli's salt. These tension changes were quantified and converted to percentage of total relaxation, and presented graphically.

The results show that greater relaxation is achieved with Angeli's salt at high concentrations (>1μM) under normal levels of glucose then under elevated levels of glucose, in both experiments with and without the HNO scavenger L-Cysteine. The lowest level of relaxation achieved was with Angeli's salt with hydroxocobalamin (HxC) with high levels of glucose present.

These results would appear to show that high glucose has the ability to inhibit HNO mediated relaxation. This would further indicate that K_V channels are involved in vasorelaxation by HNO, which concurs with data from earlier studies (Irvine *et al*, 2003; Favarolo & Kemp-Harper, 2009; Yuill *et al*, 2011; Rainbow *et al*, 2006).

Introduction

Nitroxyl (HNO) is a relatively recently discovered vasodilator, with largely speculated mechanisms of action, although it is thought to play a major role in endothelium dependant vasodilation, similar to nitric oxide (NO) (Yuill, 2011). There is some speculation that HNO may also be generated endogenously to perform this role, however there are insufficient detection methods, meaning that this cannot be conclusively proved (Paolocci *et al*, 2007; Irvine *et al*, 2008). Methods of endogenous generation of HNO that have been postulated include the oxidation of hydroxylamine (Donzelli *et al*, 2008) or the oxidation of N-hydroxy-L-arginine (Fukuto *et al*, 1992). Wong *et al* in 1998 hypothesised that the endogenous generation of HNO may be due to the reaction of S-nitrosothiols with thiols. In addition, further reaction of S-nitrosothiols with HNO can produce NO, a known vasorelaxant (Wong *et al*, 1998).

The vasodilation elicited by HNO is thought to be due to its interaction with soluble guanylate cyclase (sGC), which causes conversion of GTP to cyclic GMP (cGMP), resulting in cGMP mediated relaxation of the smooth muscle (Irvine *et al*, 2008). This mechanism has been shown in rat models with the inhibition of sGC by ODQ, which caused reduced vasodilation due to reduced levels of cGMP (Ellis *et al*, 2000; Irvine *et al*, 2007; Wanstall *et al*, 2001).

One explanation for the vasodilatory effects of HNO is the oxidation of HNO to NO by superoxide dismutase (SOD) (Liochev & Fridovich, 2002). However, uncertainty remains as to whether SOD directly oxidises HNO or whether its actions are due to the scavenging of superoxide (Zeller *et al*, 2009). One study, by Zeller *et al* (2009), concluded that the activation of sGC by HNO and the resulting vasodilation was solely due to the oxidation of HNO to NO.

However, there is also an opposing hypothesis that HNO elicits its actions in an NO independent manner. Several studies have shown that vasodilation by the actions of HNO was blocked by ODQ, a sGC inhibitor, but no blockade was achieved with the NO scavenger carboxy-PTIO, meaning there was no involvement of NO (Costa *et al*, 2001; Irvine *et al*, 2003; Irvine *et al*, 2007; Li *et al*, 1999; Wanstall *et al*, 2001).

Certain sGC independent pathways have been described for the actions of HNO. A study by Yuill *et al* in 2011 showed that high conductance calcium activated potassium (BK_{Ca}) channels were active during vasodilation with HNO, tested using specific channel inhibitors. However, there was also inhibition of vasodilation by ODQ suggesting that sGC may still be involved in this pathway. This study also ruled out oxidation of HNO to NO by using NO scavengers.

A 2003 study by Irvine *et al* showed that voltage gated potassium (K_V) channels, were activated by HNO. This study concluded that K_V channels were the only potassium channels involved, as inhibitors if this channel type blocked relaxation, and inhibitors of other potassium channel types had no effect. Open K_V channels maintain resting potentials meaning no vasoconstriction or vasodilation, but further activation of K_V channels leads to hyperpolarisation resulting in vasodilation (Rainbow *et al*, 2006). K_V channel inhibition leads to depolarisation, resulting in vasoconstriction (Rainbow *et al*, 2006).

Some studies show that the activities of K_V channels can be affected by high levels of glucose (Liu *et al*, 2001; Kinoshita *et al*, 2004). This may be due to the formation

of superoxide in response to elevated levels of glucose (Rainbow *et al*, 2006; Liu *et al*, 2001; Li *et al*, 2004). Superoxide can then interact with endogenous NO to form peroxynitrite, which can inhibit both K_V and BK_{Ca} channels, causing vasoconstriction (Rainbow *et al*, 2006; Li *et al*, 2004). In addition, inhibition of various K channels by high glucose levels appears to be dependent on protein kinase C (PKC) activation (Rainbow *et al*, 2006). It is unclear as to how elevated levels of glucose may affect vasodilation by HNO, or whether HNO interacts with superoxide or peroxynitrite. Reactive nitrogen species may also cause posttranslational modifications to channel proteins by s-nitrosylation of cysteine residues (Gonzalez *et al*, 2009).

With evidence that K_V channels are inhibited with high levels of glucose (Rainbow *et al*, 2006; Liu *et al*, 2001; Li *et al*, 2004), this study could provide further evidence of the involvement of K_V channels in HNO mediated vasodilation. It is expected that there will be reduced levels of vasodilation with elevated glucose.

It is important to know of the mechanisms elicited by increased levels of glucose, as it can have major importance in disease states, for example, in type 1 diabetes mellitus. It is clear that elevated glucose levels can cause vascular dysfunction, by inhibition of potassium channels, which can cause hypertension (Liu *et al*, 2001; Li *et al*, 2004), particularly in diabetic patients (Chukwuma, 1992; Williams, 1995; Way *et al*, 2001). This may be due to hyperglycaemia causing an increase in the PKC pathway (Rainbow *et al*, 2006; Way *et al*, 2001). Hypertension in diabetics is one of the main causes of complications including cardiovascular disease (Williams, 1995) and diabetic nephropathy (Chukwuma, 1992). Hyperglycaemia can also inhibit the actions of various vasodilators that act on potassium channels, for example, CGRP (Quayle *et al*, 1994; Aiello *et al*, 1995; Wellman *et al*, 1998).

Methods

Tissue Collection

This study uses aortic ring preparations from male Wistar rats. Animals used were killed using the approved schedule 1 methods of killing; cervical dislocation and decapitation, carried out by trained staff (Animals in Scientific Procedures Act, 1986). There were no procedures carried out prior to schedule 1 killing, and the animals were checked daily to maintain good welfare. There was no lasting harm, pain or distress to the animals. At the present time there is no alternative to using animal models to study vascular smooth muscle function. The numbers of animals required was kept to a minimum by the use of a single rat for up to 7 different studies per day, and up to 4 aorta preparations can be taken from 1 animal.

For this study the descending aorta was dissected from the Wistar rat, and was transferred to a dish containing Tyrode's solution. Then the surplus connecting tissue was stripped from the outside of the aorta, and the aorta was cut into sections of around 5mm long. The sections were only handled using forceps at the edges of the sections to avoid damaging the endothelium as far as possible. The sections of aorta were then mounted horizontally across two hooks as in figure 1.

Figure 1: Diagram showing the setup for the mounting of the aortic ring preparations

Mounting in Organ Bath

The lower hook was attached to the bottom of the reservoir, and the top hook was attached to cotton thread and connected to a calibrated isometric transducer. The transducer was connected to LabChart and was calibrated using a 1g weight. The difference between nothing attached to the transducer, and the level when the 1g weight was added was then set to 1g in the LabChart software.

The organ bath was kept in a water bath at a constant temperature of 38°C, and was filled with Tyrode's solution. The Tyrode's solution was aerated using carbogen gas to pH balance the solution.

Solutions

The physiological solution used for these experiments is Tyrode's solution. This is made up of NaCl 140mM, Glucose 5.55mM, sodium bicarbonate 11.9mM, KCl 2.68mM, NaH_2PO_4 0.4mM, $CaCl_2$ 1.84 mM and $MgCl_2$ 1.4mM. A high glucose version of Tyrode's solution was also used with the same components, but with a glucose level of 27.75mM. The drug used to constrict the aorta was phenylephrine, with a provided stock solution of 100mM, which was diluted in Tyrode's solution to make a stock of 1mM. The preliminary experiments used sodium nitroprusside (SNP) to relax the aorta. This was also provided in a 100mM stock solution, and was diluted in Tyrode's solution to produce stocks of 10µM, 100µM, 1mM and 10mM. The main experiments used Angeli's salt to relax the aorta, also at a given stock of 100mM. This was then diluted in 1mM NaOH to generate stocks of 10µM, 100µM, 1mM and 10mM. These stocks were then stored on ice to prevent self dimerisation of HNO (Fukuto & Carrington, 2011). During the main experiments, hydroxocobalamin (HxC) was used at 100µM, and L-Cysteine was used at 3mM.

SNP Dose Response

The mounted aorta section was typically mounted at a tension of around 2g, and left to equilibrate in the organ bath for around 15 minutes. A preliminary experiment was then carried out using 1 and 3µM phenylephrine to establish maximum constriction, and SNP, an NO donor, at increasing concentrations from 10nM to 30µM, to establish maximal relaxation. This produced a trace in LabChart of tension changes measured by the transducer, showing the response of the aortic section to increasing concentrations of SNP. The changes in tension following each addition of

SNP were calculated and tabulated and the percentage of maximal relaxation was determined. These percentages were then displayed graphically.

Dose Response with Angeli's Salt

For these experiments, it was established that only 1μM phenylephrine was needed to cause maximum constriction of the aorta. The initial dose response curve was generated adding increasing concentrations of Angeli's salt from 10nM to 30μM, and monitoring relaxation. Following this, dose response curves were generated in the presence of HxC, which is an NO scavenger, ensuring all results are due to HNO and not oxidation of HNO to NO or endogenously produced NO. In addition, a negative control was included, using L-Cysteine, which is an HNO scavenger and HxC, which should show whether there are any other acting mechanisms. These curves were then generated in a high glucose environment. The reservoir was washed out with Tyrode's buffer in between experiments, and then was filled with fresh buffer, to prevent contamination of results. The values for tension changes were then tabulated, and graphs produced to compare the results.

Statistical Analysis

Each trace produced had to be analysed to compare and contrast results. The average tension achieved following each addition of drugs was taken from each trace and tabulated. The differences between each addition of relaxant and the maximum constriction achieved with phenlyephrine were then calculated. The largest difference achieved with SNP was deemed to be the maximal relaxation, therefore was 100% relaxation. Using this, percentage of total relaxation was then calculated for all difference values. The mean of the percentages obtained from the same conditions was then calculated, and then the standard deviation was calculated. These data were then presented graphically with the standard deviation presented as error bars, in order to compare the effect of glucose on vasodilation.

Results

Response of aorta to SNP

The preliminary experiments show the expected rapid relaxation with increasing concentrations of SNP (Figure 2 & 3). Figure 2 shows a typical trace produced by adding 1μM and 3μM phenylephrine followed by increasing concentrations of SNP. Figure 3 was constructed by finding the differences in tension following each addition of SNP from the maximum point following phenylephrine addition. These were then converted to percentages using the largest difference as the maximal relaxation therefore 100%. This maximal relaxation value using SNP was then used as the 100% value for comparison of the Angeli's salt dose response curves.

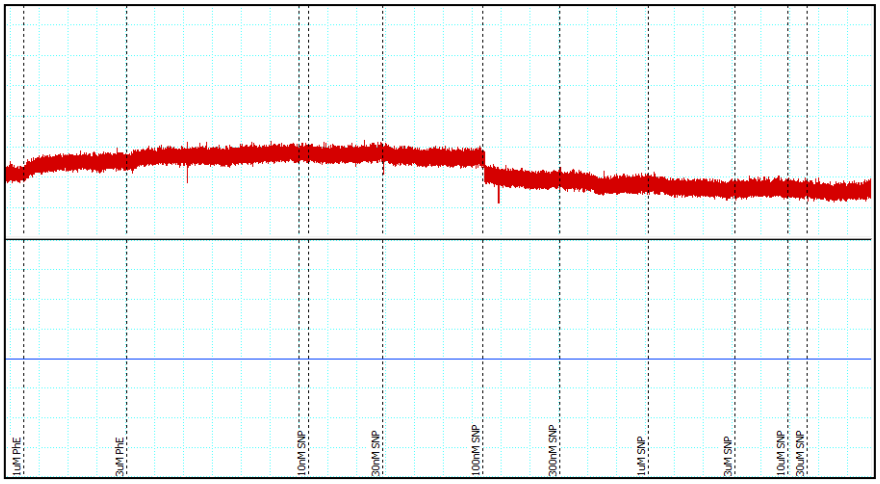

Figure 2: Typical trace produced by LabChart, showing constriction with phenylephrine and relaxation with increasing concentrations of SNP.

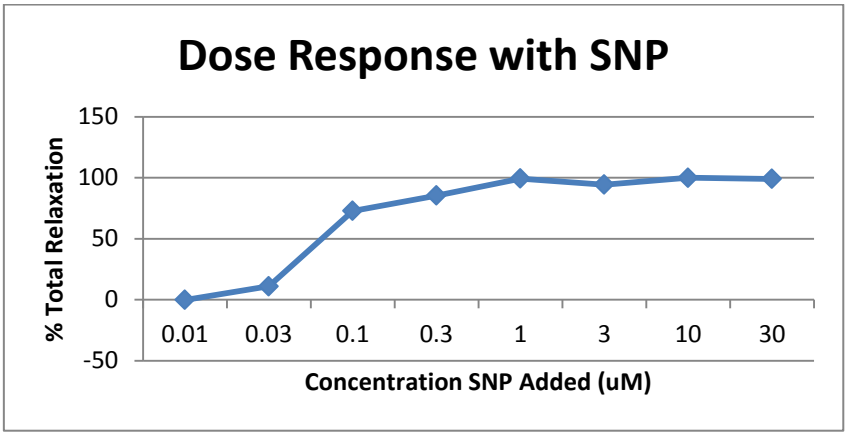

Figure 3: Graph showing the rapid relaxation with increasing concentrations

Response of aorta to Angeli's Salt

Figure 4 shows a typical LabChart trace produced under normal glucose conditions with constriction by 1μM phenylephrine, addition of the NO scavenger HxC, and relaxation by increasing concentrations of Angeli's salt. The shape of the curve at higher concentrations of Angeli's salt is due to the breakdown of the HNO in solution, stopping it from causing relaxation to the smooth muscle. Figures 5 & 6 were constructed using traces like these, by measuring the differences in tension from the maximum constriction point and each addition of Angeli's salt, just before the addition of the next increase in concentration. These differences were then converted into percentages of maximum relaxation, using the maximum with SNP as the 100% value, and then these percentages were plotted against Angeli's salt concentration.

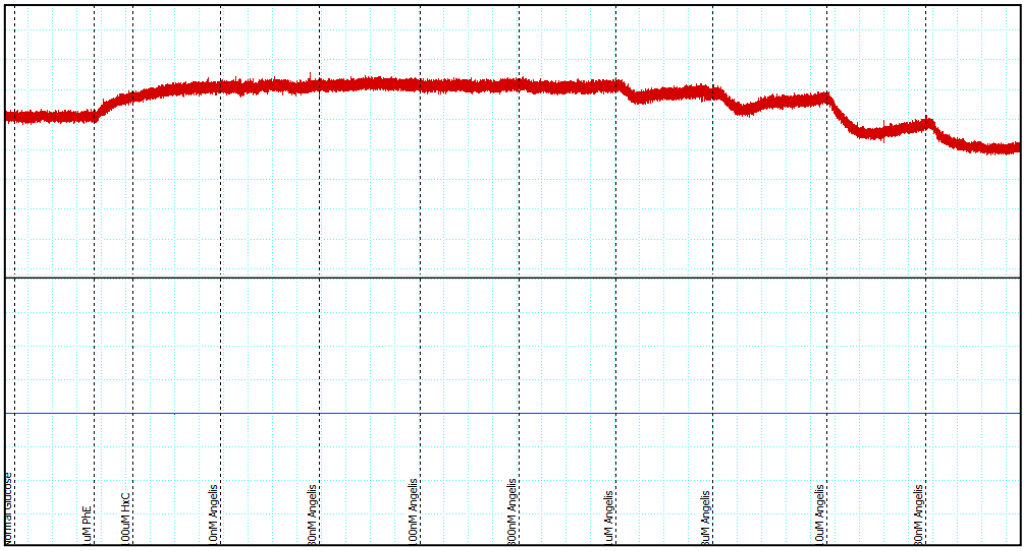

Figure 4: Typical trace produced by LabChart, showing constriction with phenylephrine and relaxation with increasing concentrations of Angeli's salt, in the presence of HxC and under normal glucose conditions.

There was only about 50% of total relaxation achieved with a concentration of 3μM of Angeli's salt (Figure 5 & 6), and then the aorta constricted slightly, so with the highest concentration of Angeli's salt (30μM), there was around 45% of total relaxation. When the NO scavenger, HxC was added, the relaxation also achieved around 45% of maximum, but there was no relaxation, and slight constriction, at the lower concentrations (<1μM) of Angeli's salt (Figure 5). Between 3-30μM additions, the relaxation was very rapid. The standard deviation was very low for the lower concentrations of Angeli's salt, indicating minimal error, but the standard deviation was very high for the higher concentrations of Angeli's salt, meaning high variability in the results.

The curve with Angeli's salt and HxC in a high glucose environment did not produce any relaxation until 3μM and then there was a small amount of relaxation up to 30μM. However the highest amount of relaxation achieved was less than 10%. The standard deviation was relatively low across these results, indicating low variability in the results (Figure 5).

The negative control used the HNO scavenger L-Cysteine in addition to Angeli's salt and HxC. This curve shows a gradual relaxation throughout additions of Angeli's salt, with a maximum relaxation achieved of around 25%. The standard deviation was low for these results, which indicates low variability (Figure 6). There appears to be more relaxation with L-Cysteine in normal glucose conditions than without L-Cysteine in high glucose conditions. The result for relaxation by Angeli's salt in the presence of HxC and L-Cysteine in high glucose conditions shows constriction until 3μM of Angeli's salt is accumulated, and eventually achieves around 25% of total relaxation (Figure 6). Both results in high glucose conditions show less relaxation than their counterparts in normal glucose conditions, and both curves generated in the presence of L-Cysteine show more relaxation than without L-Cysteine under normal glucose conditions (Figure 5 & 6).

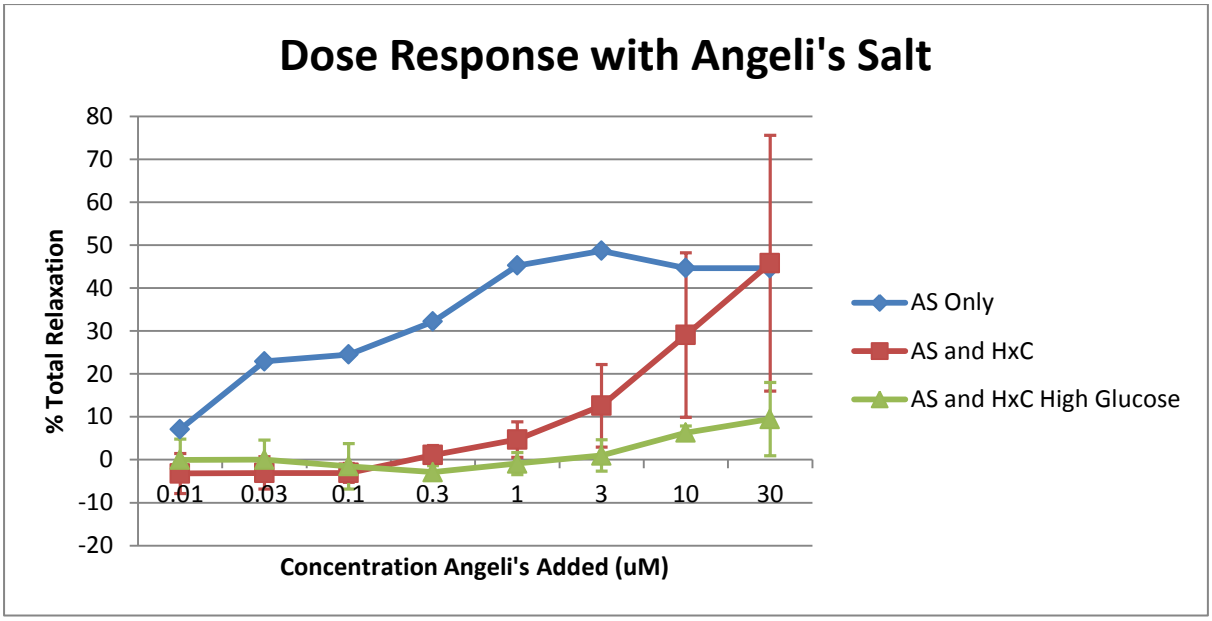

Figure 5: Graph showing mean relaxation with increasing concentrations of Angeli's salt with HxC present, with and without high glucose levels. The error bars represent the

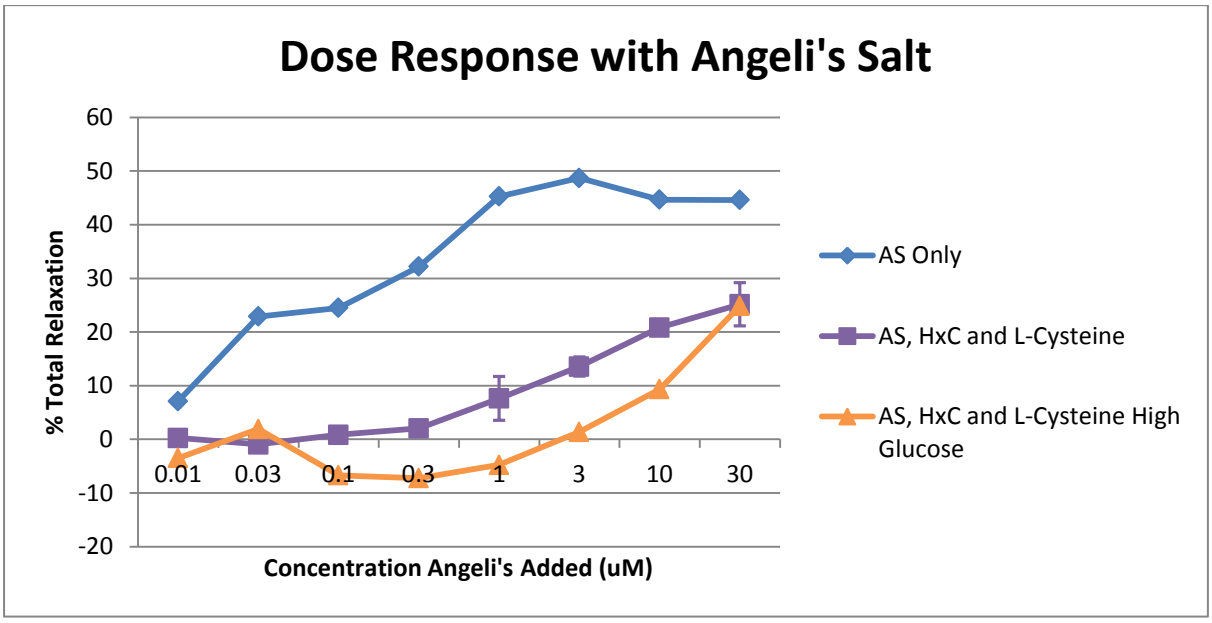

Figure 6: Graph showing mean relaxation with increasing concentrations of Angeli's salt with HxC and L-Cysteine present, with and without high glucose levels. The error bars represent the standard deviation.

Discussion

This study overall shows that high levels of glucose will reduce the ability of the aorta to relax. This indicates the involvement of K_V channels, as these channels are inhibited by high levels of glucose (Liu *et al*, 2001; Kinoshita *et al*, 2004). This was, generally, the expected result, as K_V channels have been implicated in the relaxation of smooth muscle in response to HNO (Yuill *et al*, 2011). The results obtained in this study are solely due to HNO as there was HxC used, which scavenges NO.

However, both curves generated in the presence of L-Cysteine show more relaxation than the curve without L-Cysteine in the presence of high glucose. In reality, there should be no relaxation with L-Cysteine and HxC present together, as they will scavenge HNO and NO respectively. This result could be due to the L-Cysteine that was used could be non functional, or there could be another endothelium dependent relaxation mechanism in play.

There was a large amount of variability in some of the results, particularly at higher concentrations of Angeli's salt under normal glucose conditions with HxC. It is not clear exactly what caused this inconsistency, but there are occasionally errors in preparation of the physiological buffer, in particular, with reduced calcium levels there will be reduced contractility of the smooth muscle. In addition, the carbogen gas can be variable in the pressure and volume that is used to aerate the buffer.

The shape of the curve generated in figure 5 changed when HxC was added. This is most likely due to the actions of NO at lower concentrations of Angeli's salt addition without the addition of HxC. These actions are eliminated by the scavenging actions of HxC. This result appears to agree with a 2001 study by Ellis *et al*, where the actions of HNO were found to be due to oxidation to NO, via the use of the NO scavengers, HxC and carboxy-PTIO in the aorta. This study speculated that this may be due to the oxidative environment of the aorta facilitating the oxidation of HNO to NO.

Not all values from all traces gathered were analysed for percentage graphs. This was because on some occasions there was no reaction from the aorta when either phenylephrine, SNP or Angeli's salt were added. These data were excluded as it would introduce more variability and error into the results, and would have no beneficial effect to either prove or disprove the hypothesis.

This is a relatively simple study that is easy to replicate, and can be very adaptable. Minimal damage was made to the endothelium as all sections of aorta were carefully handled. In the statistical analysis, the difference between the tension when the constriction agent, phenylephrine, was added, and the tensions when each concentration of Angeli's salt was added were taken, and were converted to percentages of maximum relaxation. This then cancels out the variability in the baseline tension, before any agent was added. There was also consistency kept in the experimental procedures, as the organ bath reservoir was kept in a temperature controlled water bath at 38°C, and the same volume of physiological buffer was used within the reservoir each time. The reservoir was washed out after each experiment to prevent the drugs used from impacting on the next test.

Some of the main drawbacks of this type of study include that it cannot show how HNO will work in vivo, as there will be trauma to the tissues when dissected, and it is conducted under very controlled conditions that won't be present in vivo. This study

will also not provide specific information as to the mechanism of how HNO is working and how glucose specifically interacts with channels or receptors in situations with high levels of glucose. This means the exact mechanisms of action can only be speculated at this point. The main method by which it can be proven that K_V channels are involved is via patch clamp experiments, which will show changes in current, and have been used in a number of studies (Li *et al*, 2004; Yuill *et al*, 2011). Another drawback of this study is that the increased glucose concentration may have changed the osmolarity of the buffer, which can affect how the drugs perform.

The findings of this study appear to agree with those of previous studies. This study has found that it is likely that K_V channels are involved in HNO mediated vasodilation, as the relaxation is inhibited by glucose, and it has been shown that high levels of glucose affect the actions of K_V channels (Rainbow *et al*, 2006). Previous studies that have used different methods to those in this study have shown that sGC is produced when HNO is administered, and activates K_V channels to mediate an HNO concentration dependant relaxation (Irvine *et al*, 2003; Favarolo & Kemp-Harper, 2009; Yuill *et al*, 2011).

Further research is needed to establish the exact interactions between HNO and glucose, in particular, whether glucose can affect HNO directly or whether there are other interactions at play. It could also prove useful to compare the effect of elevated levels of glucose on HNO and NO, as it is known how glucose inhibition of K_V channels occurs with NO (by the inhibitory effects of peroxynitrite generated by reaction of superoxide with NO) (Rainbow *et al*, 2006; Li *et al*, 2004), but the mechanisms surrounding HNO are unknown. The methods in this study could also be built upon by testing at a range of environmental glucose concentrations, and possibly testing with no glucose. Testing various glucose levels may be able to provide useful information that could be pertinent to diabetic patients, as hyperglycaemia can lead to hypertension, which underlies many of the complications of diabetes (Chukwuma, 1992; Williams, 1995; Way *et al*, 2001). It could prove useful to know how severe the hyperglycaemia has to be to cause inhibition of vasorelaxation, hypertension, and ultimately complications arising from this. It may also be useful to test with ODQ to inhibit sGC to find out whether the mechanisms by which glucose inhibits potassium channels are sGC dependent or independent, as it has been shown that under normal glucose conditions, HNO activates sGC to cause activation of K_V channels (Irvine *et al*, 2003). In addition, specific channel blockers could be used to pinpoint the exact channels involved in vasorelaxation in response to HNO, and involved in inhibition of relaxation by glucose. Glucose levels may also me important in terms of cardioprotection from ischemic injury. It has been shown that HNO can increase the activity of ATP dependant potassium (K_{ATP}) channels conferring cardioprotection (Queliconi *et al*, 2011), however, increased levels of glucose may cause a down regulation of the Kir6.2 component of the K_{ATP} channel (Moritz *et al*, 2001), which would affect cardioprotection by HNO.

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